

In Vivo Activation of Midbrain Dopamine Neurons via Sensitized, High-Affinity $\alpha 6^*$ Nicotinic Acetylcholine Receptors

Ryan M. Drenan,¹ Sharon R. Grady,² Paul Whiteaker,^{2,6} Tristan McClure-Begley,² Sheri McKinney,¹ Julie M. Miwa,³ Sujata Bupp,³ Nathaniel Heintz,³ J. Michael McIntosh,⁴ Merouane Bencherif,⁵ Michael J. Marks,² and Henry A. Lester^{1,*}

¹Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

²Institute for Behavioral Genetics, University of Colorado Boulder, Boulder, CO 80309, USA

³The Laboratory of Molecular Biology, Howard Hughes Medical Institute, Rockefeller University, New York, NY 10021, USA

⁴Departments of Psychiatry and Biology, University of Utah, Salt Lake City, UT 84112, USA

⁵Department of Preclinical Research, Targacept Inc., Winston-Salem, NC 27101, USA

⁶Present address: Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013, USA

*Correspondence: lester@caltech.edu

DOI 10.1016/j.neuron.2008.09.009

SUMMARY

$\alpha 6$ -containing ($\alpha 6^*$) nicotinic ACh receptors (nAChRs) are selectively expressed in dopamine (DA) neurons and participate in cholinergic transmission. We generated and studied mice with gain-of-function $\alpha 6^*$ nAChRs, which isolate and amplify cholinergic control of DA transmission. In contrast to gene knockouts or pharmacological blockers, which show necessity, we show that activating $\alpha 6^*$ nAChRs and DA neurons is sufficient to cause locomotor hyperactivity. $\alpha 6^{L9/S}$ mice are hyperactive in their home cage and fail to habituate to a novel environment. Selective activation of $\alpha 6^*$ nAChRs with low doses of nicotine, by stimulating DA but not GABA neurons, exaggerates these phenotypes and produces a hyperdopaminergic state in vivo. Experiments with additional nicotinic drugs show that altering agonist efficacy at $\alpha 6^*$ provides fine tuning of DA release and locomotor responses. $\alpha 6^*$ -specific agonists or antagonists may, by targeting endogenous cholinergic mechanisms in midbrain or striatum, provide a method for manipulating DA transmission in neural disorders.

INTRODUCTION

Identification of the relevant nAChRs involved in (1) normal DA transmission, (2) disorders of the DA system, such as schizophrenia, Parkinson's disease, and ADHD, and (3) nicotine dependence is a major priority. $\alpha 6^*$ (* indicates that other subunits may be present in the pentameric receptor) nAChRs are highly and selectively expressed in DA neurons, with additional functional expression in locus coeruleus and retinal ganglion cells (Azam and McIntosh, 2006; Azam et al., 2002; Champtiaux et al., 2002; Gotti et al., 2005b; Le Novère et al., 1996). $\alpha 6^*$ nAChRs are selectively inhibited by the marine cone snail peptide α -conotoxin MII (α CtxMII) (Cartier et al., 1996; Whiteaker et al.,

2000). Immunoprecipitation and α CtxMII-binding studies demonstrated that $\alpha 6\beta 2\beta 3^*$ and $\alpha 6\alpha 4\beta 2\beta 3^*$ pentamers are the predominant $\alpha 6^*$ nAChRs in striatum (Champtiaux et al., 2003; Zoli et al., 2002). $\alpha 6\beta 2^*$ receptors account for ~30% of nicotine-stimulated DA release in striatum (Grady et al., 2002; Kaiser et al., 1998; Kulak et al., 1997). $\beta 3$ subunits are encoded by a gene adjacent to *Chrna6*, are usually coexpressed with $\alpha 6$, and are essential for $\alpha 6^*$ nAChR biogenesis and function (Cui et al., 2003; Gotti et al., 2005a). $\alpha 6^*$ receptors exhibit the highest known sensitivity to nicotine and ACh in functional measurements on native receptors (Salminen et al., 2004) yet function poorly in heterologous expression systems (Drenan et al., 2008). As a result, there has been little progress in defining selective agonists for $\alpha 6^*$ nAChRs or on other functional measurements. Furthermore, in midbrain DA neurons, studies of somatodendritic $\alpha 6^*$ receptors are complicated by the presence of $\alpha 4\beta 2^*$ (non- $\alpha 6$), and selective antagonists of $\alpha 4\beta 2^*$ (non- $\alpha 6$) have not been identified.

Many experiments with selective destruction of DA neurons show that the activity of such neurons is necessary for reinforcement of natural and artificial rewards. These cells exhibit tonic and phasic firing patterns (Grenhoff et al., 1986), where phasic or "burst" firing carries salient information thought to predict imminent reward status (Schultz, 2002). Pedunculopontine tegmentum (PPTg) and laterodorsal tegmentum (LDTg) fibers provide a cholinergic drive that strongly regulates DA neuron excitability and the transition to burst firing (Lanca et al., 2000). Midbrain nAChRs in three locations respond to mesopontine-derived ACh: (1) $\alpha 7^*$ nAChRs on glutamatergic terminals from cerebral cortex, (2) $\alpha 4\beta 2^*$ nAChRs on GABAergic terminals and cell bodies, and (3) $\alpha 4^*$ and $\alpha 6^*$ somatodendritic nAChRs on DA neurons (Calabresi et al., 1989). Nicotine interferes with normal cholinergic transmission to DA neurons, in part by modifying the weights of these various nAChR synapses. For example, nicotine at concentrations found in the CSF of smokers preferentially desensitizes $\alpha 4\beta 2^*$ nAChRs regulating midbrain GABA release (Mansvelder et al., 2002) yet still permits $\alpha 7^*$ nAChR-regulated glutamate release (Mansvelder and McGehee, 2000). This produces both disinhibition and direct excitation of DA neurons, increasing the probability of a switch to burst firing.

Nicotine also exaggerates the action of endogenous ACh in regulating DA release in striatum. Striatal cholinergic interneurons continually release ACh that activates nAChRs, which maintains background DA levels during tonic firing of midbrain DA neurons (Zhou et al., 2001). However, DA release in response to burst firing of DA neurons is facilitated by a reduction in nAChR activity (Rice and Cragg, 2004; Zhang and Sulzer, 2004). $\alpha 6^*$ receptors, due to their high sensitivity and their selective expression in DA cell bodies and presynaptic terminals, are probably key players in cholinergic control of DA release. We reasoned that genetic “sensitization” of these receptors would (1) amplify the role of $\alpha 6^*$ nAChRs in cholinergic control of DA transmission and (2) allow for selective pharmacological stimulation of these neurons. This approach would complement previous experiments using $\alpha 6$ knockout mice and $\alpha 6^*$ pharmacological blockade, demonstrating behavioral and physiological responses for which $\alpha 6^*$ nAChRs are sufficient.

We introduced a bacterial artificial chromosome (BAC) transgene into the mouse germline with a mutant copy of the mouse *Chrna6* gene that rendered mutant $\alpha 6^*$ channels “hypersensitive” to endogenous ACh or exogenous nicotine. DA neuron excitability and DA release is greatly augmented in these mice, and they exhibit behavioral phenotypes consistent with increased DA neuron firing and/or DA release. These studies improve our knowledge of cholinergic regulation of the midbrain DA system and of $\alpha 6^*$ nAChR biology and have implications for the treatment of disorders involving changes in DA.

RESULTS

Production and Characterization of BAC $\alpha 6^{L9/S}$ Transgenic Mice

We selected a 156 kb mouse BAC clone containing the genomic *Chrna6* ($\alpha 6$ nAChR) locus with substantial 5' and 3' flanking genomic regions for generating a targeting construct to faithfully recapitulate expression of *Chrna6*. $\alpha 6$ Leu280 (the Leu 9' residue in the M2 domain) was mutated to Ser via homologous recombination using a two-step selection/counterselection procedure in *E. coli* (Figure S1A available online). The final construct was injected into fertilized mouse eggs, and six transgenic offspring were identified by genomic DNA sequencing and diagnostic PCR (Figure S1B and S1C). To eliminate possible artifacts of transgene position/insertion, we analyzed two independently derived lines (lines 2 and 5), which have different transgene copy numbers (Figure S1D) and different genomic positions. Both mouse lines expressed mutant $\alpha 6^{L9/S}$ mRNA in addition to wild-type (WT) $\alpha 6$ mRNA (Figure S1E).

We found no difference in location or intensity of [125 I]- α -conotoxin MII (α CtxMII) labeling in $\alpha 6^{L9/S}$ versus WT brains, confirming correct regional expression of $\alpha 6^*$ nAChRs in mutant mice (Figure S1F). To corroborate this, we quantified $\alpha 6^*$ -binding sites. Membranes were prepared from striatum (ST), olfactory tubercle (OT), superior colliculus (SC) (regions that account for most $\alpha 6^*$ -binding sites), and thalamus (TH) (where most binding sites are $\alpha 4\beta 2^*$). [125 I]-epibatidine binding, coupled with inhibition by unlabeled α CtxMII, revealed $\alpha 6^*$ and non- $\alpha 6^*$ ($\alpha 4\beta 2^*$) receptors (Figure S1G). Collectively, these results indicate that $\alpha 6^{L9/S}$ mice exhibit normal levels and localization of neuronal nAChRs.

Spontaneous and Nicotine-Induced Hyperactivity in $\alpha 6^{L9/S}$ Mice

We measured home cage horizontal locomotor activity for WT and $\alpha 6^{L9/S}$ mice over a period of 48 hr. $\alpha 6^{L9/S}$ mice were markedly hyperactive relative to WT control littermates (Figure 1A). This effect was restricted to lights off (Figure 1B), though there was a nonsignificant trend toward hyperactivity during lights on. Although WT mice show locomotor habituation when placed into a novel environment, $\alpha 6^{L9/S}$ mice exhibit sustained activity. We measured WT and $\alpha 6^{L9/S}$ locomotor activity for 30 min after placement in a new home cage environment (Figure 1C). Activity during the first 15 min of the session was unchanged, but from $t = 16$ to 30 min, $\alpha 6^{L9/S}$ mice exhibited significantly greater activity than WT littermates (Figure 1D).

$\alpha 6^*$ nAChRs are highly expressed in DAergic neurons, and nicotine has psychomotor stimulant properties in rodents. We injected WT and $\alpha 6^{L9/S}$ mice with nicotine and measured locomotor activity. Low doses of nicotine (0.15 mg/kg, i.p.) strongly activated locomotion in $\alpha 6^{L9/S}$ mice but had no effect in WT mice (Figure 1E). To characterize the locomotor activation phenotype in $\alpha 6^{L9/S}$ mice, we constructed a nicotine dose-response relation. WT mice exhibited locomotor suppression at doses of nicotine between 0.5 and 2.0 mg/kg, i.p. (Figure 1F), consistent with previous work (Tapper et al., 2007). In contrast, $\alpha 6^{L9/S}$ mice exhibited steadily increasing locomotor responses between 0.02 and 0.15 mg/kg, i.p. nicotine, followed by a decline at 0.4 mg/kg, i.p. and locomotor suppression similar to WT mice at 1.5 mg/kg, i.p. Thus, selective activation of $\alpha 6^*$ receptors results in locomotor activation rather than locomotor suppression. This phenotype was not a stress response, as saline injections did not produce locomotor activation (Figure 1G).

Locomotor activation in $\alpha 6^{L9/S}$ mice was dependent on activation of nicotinic receptors; we noted a strong block of the locomotor response in $\alpha 6^{L9/S}$ (but not WT) mice preinjected with mecamylamine (1 mg/kg, i.p.) (Figure 1H). Further, $\alpha 6^{L9/S}$ locomotor activation acted through dopamine receptors; the response to 0.15 mg/kg, i.p. nicotine was completely inhibited by SCH23390 (D1DR antagonist; 2 mg/kg, i.p.) and partially inhibited by sulpiride (D2DR antagonist; 20 mg/kg, i.p.) (Figure 1H).

To determine whether $\alpha 6^{L9/S}$ mice develop tolerance or sensitization to nicotine psychomotor stimulation, we injected groups of $\alpha 6^{L9/S}$ mice once daily for 6 days with nicotine. Injection of 0.02 mg/kg, i.p. or 0.08 mg/kg, i.p. nicotine did not produce any change in locomotor behavior after repeated injections (Figure 1I). Nicotine also produces hypothermia in mice, and $\alpha 4^*$ hypersensitive mice exhibit this effect at nicotine doses ~50-fold lower than WT mice (Tapper et al., 2004). We found no difference between the hypothermia responses of WT and $\alpha 6^{L9/S}$ mice (data not shown).

Augmented Nicotine-Stimulated DA Release from Presynaptic Terminals in $\alpha 6^{L9/S}$ Mice

Mouse $\alpha 6^*$ nAChRs expressed in midbrain DA neurons are located both on the cell body and on presynaptic terminals in caudate/putamen (CPu), nucleus accumbens (NAc), and striatal aspects of the olfactory tubercle (OT). We measured nicotine-stimulated [3 H]DA release from striatal synaptosomes of WT and $\alpha 6^{L9/S}$ mice. We made separate tissue samples containing

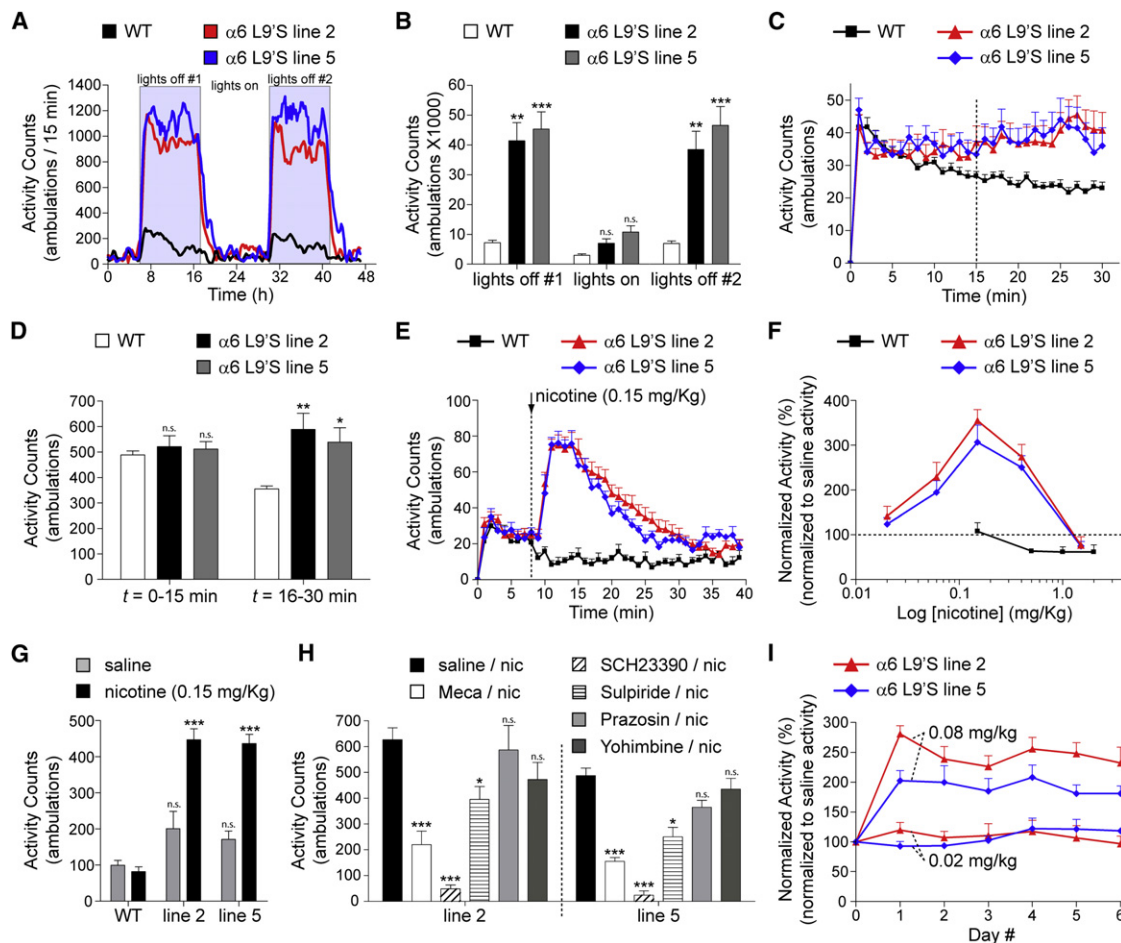


Figure 1. $\alpha 6^{L9/S}$ Mice Are Markedly Hyperactive and Hypersensitive to the Psychomotor Stimulant Action of Nicotine

(A and B) $\alpha 6^{L9/S}$ mice are hyperactive in their home cage. Horizontal locomotor activity of mice ($\alpha 6^{L9/S}$ or WT cagemates) in their home cage environment was recorded during a 48 hr period. Raw locomotor activity data (number of ambulations/15 min period) are reported (A). Total locomotor activity from "lights on" and "lights off" periods indicated in (A) are shown (B) for WT and $\alpha 6^{L9/S}$ mice.

(C and D) $\alpha 6^{L9/S}$ mice do not habituate to a novel environment. Mice ($\alpha 6^{L9/S}$ or WT cagemates) were removed from their home cage and immediately placed in a fresh cage. Locomotor activity was measured for 30 min. Raw locomotor activity data (number of ambulations/min) are reported (C). Total locomotor activity from (C) for $t = 0-15$ min or $t = 16-30$ min is shown (D).

(E) Nicotine specifically stimulates locomotor activity in $\alpha 6^{L9/S}$ transgenic mice. After 8 min of baseline locomotor activity, mice (WT, $\alpha 6^{L9/S}$ line 2 and 5) were injected with 0.15 mg/kg i.p. nicotine. Locomotor activity was recorded for an additional 30 min after injection. Raw locomotor activity data (number of ambulations/min) are reported.

(F) Dose-response relationship for nicotine-stimulated locomotor activity in WT and $\alpha 6^{L9/S}$ mice. Mice were administered nicotine at the indicated dose, and total locomotor activity was measured as in (E). Locomotor activity for each mouse was normalized to a saline control injection for the same mouse. Data are expressed as the percentage of the response to saline (set to 100%).

(G) Locomotor activity following saline or nicotine (0.15 mg/kg, i.p.) injection in WT and $\alpha 6^{L9/S}$ mice. Total raw locomotor activity data (number of ambulations/min, 30 min total) are reported.

(H) Behavioral pharmacology of nicotine-stimulated locomotor activation in $\alpha 6^{L9/S}$ mice. Mice (WT or $\alpha 6^{L9/S}$) were preinjected with saline, mecamylamine (1 mg/kg, i.p.), SCH23390 (2 mg/kg, i.p.), sulpiride (20 mg/kg, i.p.), prazosin (1 mg/kg, i.p.), or yohimbine (1 mg/kg, i.p.) immediately prior to the start of data acquisition. Nicotine (0.15 mg/kg, i.p.) was injected at $t = 8$ min, and locomotor activity was measured for an additional 30 min. Total raw locomotor activity data (number of ambulations/min, 30 min total) are reported.

(I) Lack of sensitization or tolerance following repeated nicotine administration in $\alpha 6^{L9/S}$ mice. Groups of $\alpha 6^{L9/S}$ mice were injected with saline once daily for 3 consecutive days, followed by nicotine (0.02 mg/kg, i.p., or 0.08 mg/kg, i.p.) once daily for 6 consecutive days. Locomotor activity was measured as in (E), and nicotine-stimulated locomotor activity for each mouse was normalized to saline control injections (average of three saline injections) for the same mouse. Data are expressed as the percentage of the response to saline (set to 100%).

All data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

striatum (ST; CPU and dorsal aspects of NAc) and olfactory tubercle (OT). Because CPU receives DAergic projections mainly from substantia nigra whereas OT receives DAergic projections

exclusively from VTA, this preparation crudely separates the mesostriatal and mesolimbic pathways. For total DA release in ST, there was no difference in R_{max} (Figures 2A and S2) and

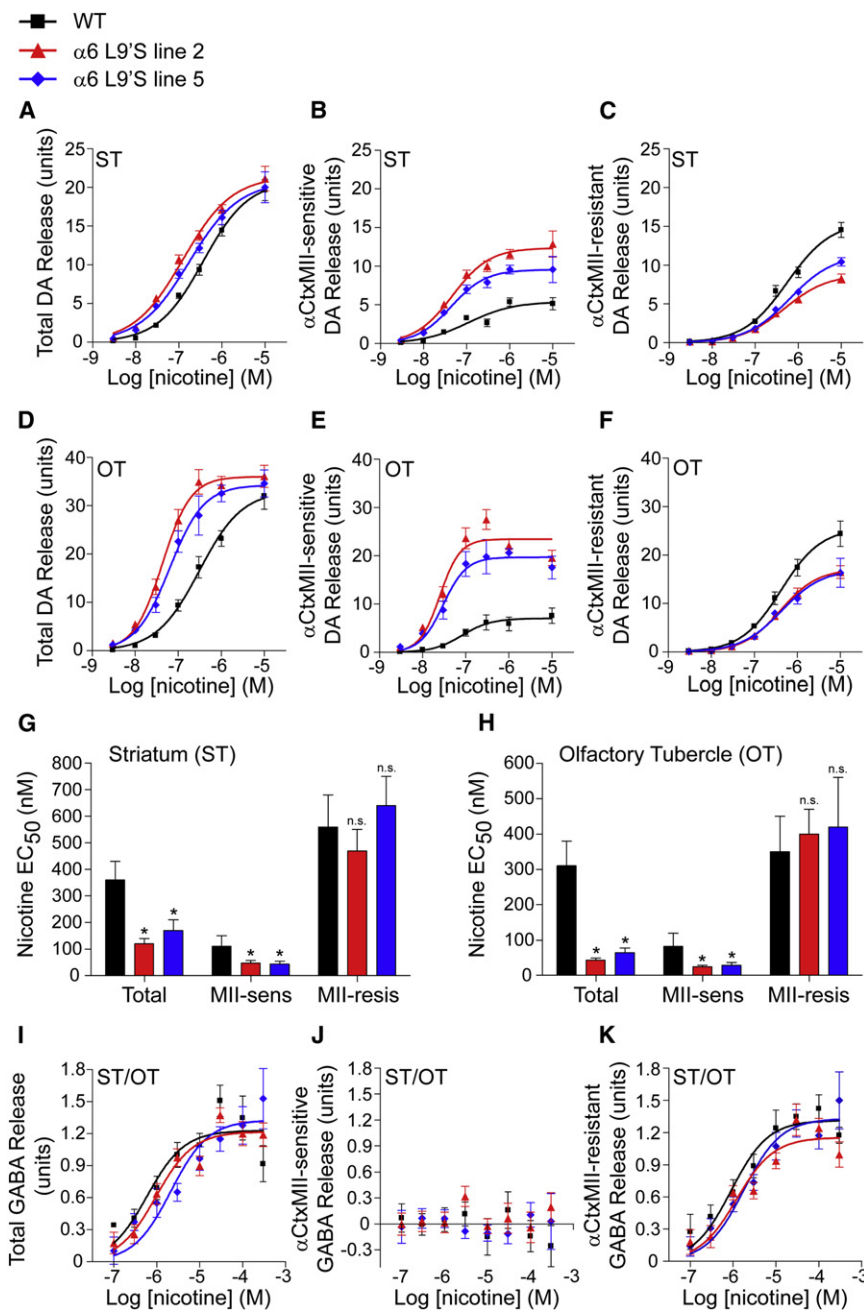


Figure 2. Presynaptic $\alpha 6^*$ nAChRs in Striatum Mediate Hypersensitive, Nicotine-Stimulated DA Release in $\alpha 6^{L9/S}$ Transgenic Mice

(A–C) Hypersensitive DA release in striatum of $\alpha 6^{L9/S}$ transgenic mice is $\alpha 6^*$ dependent. Striata (“ST”; dorsal striatum and dorsal aspects of nucleus accumbens) from WT and $\alpha 6^{L9/S}$ mice were dissected and synaptosomes were prepared. DA release was stimulated with a range of nicotine concentrations (3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μ M, 10 μ M), and a concentration-response relation for each mouse line ($n = 6$ mice/line) is shown for total release (A). To determine the relative contribution of $\alpha 6^*$ and non- $\alpha 6^*$ receptors, striatal synaptosome samples were incubated with α CtxMII (50 nM). $\alpha 6^*$ -independent release is shown in (C), and α CtxMII-sensitive ($\alpha 6^*$ dependent) release is shown in (B). (D–F) Hypersensitive DA release in olfactory tubercle of $\alpha 6^{L9/S}$ transgenic mice is $\alpha 6^*$ dependent. Olfactory tubercle (“OT”) was dissected from WT and $\alpha 6^{L9/S}$ mice, and samples were processed as in (A)–(C).

(G and H) Quantification of hypersensitive DA release in striatum (G) and olfactory tubercle (H). Average nicotine EC_{50} values for each concentration-response curve from (A)–(F) are shown.

(I–K) $\alpha 6^*$ nAChRs do not participate in nicotine-stimulated GABA release in striatum. ST and OT were dissected and combined, followed by synaptosome preparation. GABA release was stimulated by nicotine at the following concentrations: 100 nM, 300 nM, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 100 μ M, and 300 μ M. Total (I) release and α CtxMII-sensitive (J) and resistant (K) components are shown.

Data are expressed as mean \pm SEM. * $p < 0.05$.

change in EC_{50} for the non- $\alpha 6$ component (Figures 2G and 2H). Overall, DA release from $\alpha 6^{L9/S}$ striatal synaptosomes may be underestimated by this assay, as 20 mM K^+ stimulated slightly less DA release in both $\alpha 6^{L9/S}$ lines relative to WT mice (Figure S2). These results directly reveal that selective $\alpha 6^*$ activation is capable of stimulating striatal DA release.

$\alpha 4\beta 2^*$ nAChRs modulate striatal GABA release (Lu et al., 1998). We measured

a small but significant reduction in EC_{50} for both transgenic lines relative to WT (Figure 2G). In OT, there was a slight increase in R_{max} (Figures 2D and S2) and a greater reduction in the EC_{50} for $\alpha 6^{L9/S}$ lines compared to WT (Figure 2H). We used α CtxMII to inhibit $\alpha 6^*$ receptors, revealing the contribution of $\alpha 6^*$ and non- $\alpha 6^*$ nAChRs to this augmented DA release. In ST and OT, we observed a significant increase in total DA release mediated by $\alpha 6$ at most nicotine concentrations (Figures 2B and 2E). Interestingly, this was accompanied by a concomitant decrease in the non- $\alpha 6^*$ ($\alpha 4\beta 2^*$ mediated) component (Figures 2C and 2F) in $\alpha 6^{L9/S}$ samples. In ST and OT, we measured a significant reduction in the EC_{50} for the $\alpha 6$ -dependent component and no

GABA release from striatal (ST and OT combined) synaptosomes of WT and $\alpha 6^{L9/S}$ mice to determine whether any $\alpha 6^*$ -dependent component was revealed by the gain-of-function L9/S mutation. There was no difference in total GABA release for any genotype comparison (Figures 2I and S3), and there was no $\alpha 6^*$ component (Figures 2J, 2K, and S3). A Student's t test on R_{max} and EC_{50} values revealed no significant difference between total and α CtxMII-resistant GABA release for any genotype (WT R_{max} $p = 0.47$, WT EC_{50} $p = 0.73$; line 2 R_{max} $p = 0.93$, line 2 EC_{50} $p = 0.31$; line 5 R_{max} $p = 0.62$, line 5 EC_{50} $p = 0.63$). Furthermore, there was no significant difference between any genotype on R_{max} or EC_{50} (two-way ANOVA with Tukey post

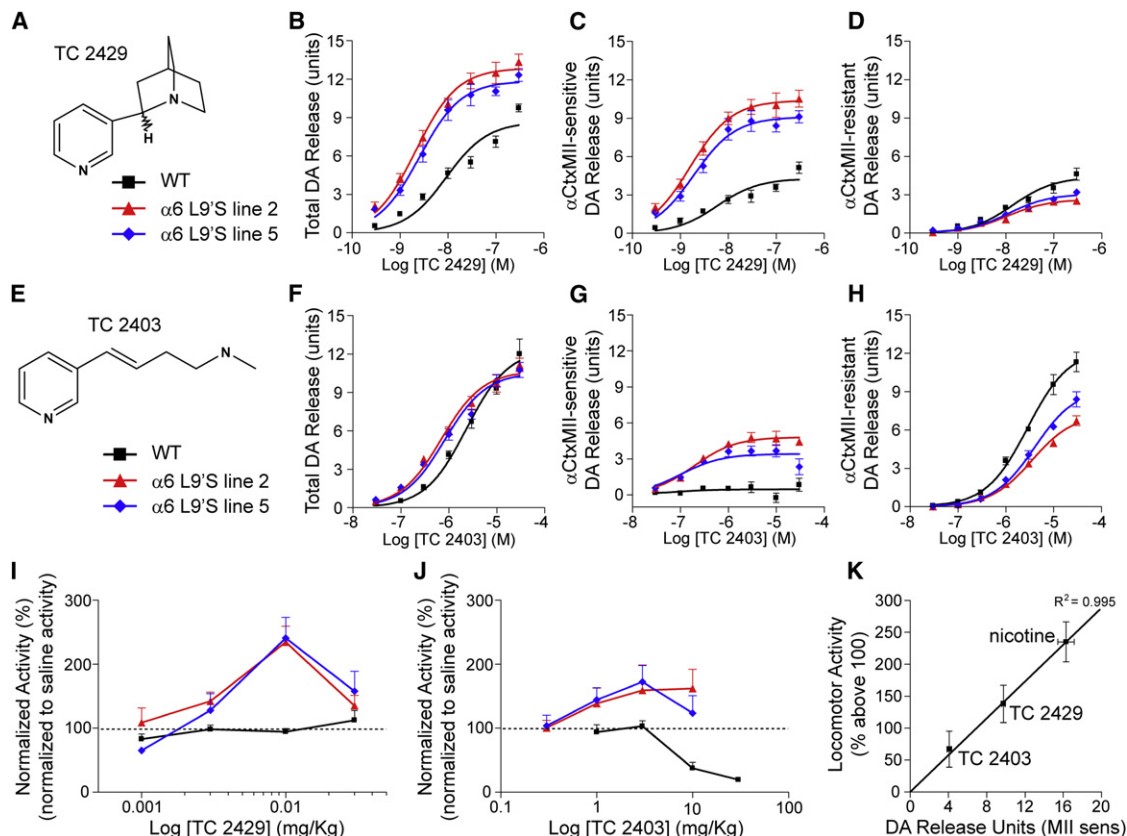


Figure 3. $\alpha 6$ -Dependent Neurotransmitter Release and Behavioral Phenotypes Are Discriminated by Subtype-Selective Nicotinic Compounds

(A) TC 2429 structure.

(B–D) An $\alpha 6^+$ -selective nicotinic agonist demonstrates enhanced striatal DA release in $\alpha 6^{L9'S}$ mice. Striatum (ST) and olfactory tubercle (OT) from WT and $\alpha 6^{L9'S}$ mice were dissected and combined, followed by synaptosome preparation. DA release was stimulated with a range of nicotine concentrations (see Figure 2A), and total (B) release as well as α CtxMII-sensitive (C) and α CtxMII-resistant (D) components are shown for each genotype.

(E) TC 2403 structure.

(F–H) An $\alpha 4^+$ -selective nicotinic agonist modestly activates striatal $\alpha 6^+$ nAChRs in $\alpha 6^{L9'S}$ mice. Similar to (B)–(D), total DA release (F) as well as α CtxMII-sensitive (G) and α CtxMII-resistant (H) components are shown for TC 2403.

(I) An $\alpha 6^+$ -selective agonist stimulates locomotor activity in $\alpha 6^{L9'S}$ mice without affecting WT activity. Mice were administered TC 2429 at the indicated dose, and total locomotor activity was measured as in Figure 1. Locomotor activity for each mouse was normalized to saline control injections in the same mouse.

(J) $\alpha 6^+$ nAChR stimulation by TC 2403 induces modest locomotor activity in $\alpha 6^{L9'S}$ mice. Mice were administered TC 2403 at the indicated dose, and total locomotor activity was measured as in Figure 1. Locomotor activity for each mouse was normalized to saline control injections in the same mouse.

(K) Close correlation between $\alpha 6^+$ -mediated DA release and locomotor activity. For nicotine (Figure 2), TC 2429, and TC 2403, α CtxMII-sensitive DA release average R_{max} values for lines 2 and 5 (ST and OT combined) were plotted versus peak locomotor activity (percentage above 100). A regression line ($R^2 = 0.995$) for the three compounds is shown.

Data are reported as mean \pm SEM.

hoc comparison: R_{max} $F_{(2,66)} = 1.87$, $p = 0.163$; EC_{50} $F_{(2,66)} = 0.744$, $p = 0.48$) nor was there any effect of α CtxMII across all genotypes on R_{max} or EC_{50} (R_{max} $F_{(1,66)} = 0.35$, $p = 0.554$; EC_{50} $F_{(1,66)} = 0.643$, $p = 0.426$). Stimulation with 20 mM K^+ showed no differences between WT and $\alpha 6^{L9'S}$ samples for GABA release (Figure S3). This indicates that GABAergic terminals in striatum, either derived locally or from VTA or SNr GABAergic neurons, contain no appreciable $\alpha 6^+$ nAChRs.

$\alpha 6^+$ receptors synthesized in retinal ganglion cells reside in the superficial layers of superior colliculus (SC) (Gotti et al., 2005b), and we measured nicotine-stimulated $^{86}Rb^+$ efflux from SC synaptosomes. $\alpha 6^{L9'S}$ SC α CtxMII-sensitive receptors are hyper-

sensitive to nicotine relative to WT, whereas α CtxMII-resistant $^{86}Rb^+$ efflux is unchanged (Figure S4).

DA Release and Locomotor Activity Are Precisely Controlled by Varying $\alpha 6^+$ Agonist Efficacy

The DA release data suggest a mechanism involving dopamine for the psychomotor stimulant action of nicotine as well as the spontaneous hyperactivity observed. For DA release in WT mice, TC 2429 (Figure 3A) (Bhatti et al., 2008) is a full agonist (versus nicotine) with 3-fold selectivity at $\alpha 6\beta 2^+$ and a weak partial agonist at $\alpha 4\beta 2$, whereas TC 2403 (Figure 3E) (Bencherif et al., 1996; Lippiello et al., 1996) is a full agonist (versus nicotine) at

$\alpha 4\beta 2$ and has no activity at $\alpha 6\beta 2^*$ (Figure S5). TC 2429, like nicotine, was more efficacious and more potent for DA release from striatal synaptosomes of $\alpha 6^{L9/S}$ mice relative to WT (Figure 3B). In both $\alpha 6^{L9/S}$ lines, R_{\max} was greater, and the EC_{50} was reduced relative to WT. This was entirely attributable to $\alpha 6^*$ nAChRs (Figure 3C), which accounted for a greater proportion of the total response. There was a concomitant decline in the total response but not the EC_{50} for $\alpha 4\beta 2^*$ (α CtxMII resistant) nAChRs (Figure 3D). We characterized the ability of TC 2429 to induce psychomotor activation in $\alpha 6^{L9/S}$ and WT control mice. Similar to nicotine, injections of TC 2429 stimulated locomotor activity in $\alpha 6^{L9/S}$ mice but not WT (Figure 3I). Unlike nicotine, TC 2429 did not produce locomotor suppression in WT mice.

TC 2403 was slightly more potent and had equivalent efficacy for DA release in $\alpha 6^{L9/S}$ versus WT striatum (Figure 3F). The increased potency was due to the amplification of an α CtxMII-sensitive response not visible in WT tissue preparations (Figure 3G). TC 2403 was a partial agonist at this receptor (Figure S5). Consistent with competition between $\alpha 6$ and $\alpha 4$ subunits for common $\beta 2$ subunits, there was a decline in R_{\max} in $\alpha 6^{L9/S}$ mice for $\alpha 4\beta 2^*$ (α CtxMII resistant) nAChRs (Figures 3H and S5). In locomotor assays, TC 2403 induced a slight locomotor activation in $\alpha 6^{L9/S}$ but not WT mice (Figure 3J), consistent with partial activity at $\alpha 6^*$ nAChRs. Nicotine (10 μ M) was used as a positive control for DA release for TC 2429 and TC 2403 (Figure S5). For nicotine, TC 2429, and TC 2403, we noted a tight correlation between $\alpha 6^*$ -dependent DA release in vitro and peak locomotor activity in vivo (Figure 3K). These results suggest a mechanism whereby agonism at $\alpha 6^*$ nAChRs stimulates striatal DA release and produces locomotor stimulation, perhaps without GABAergic attenuation, which is normally coactivated by $\alpha 4\beta 2^*$ activation (Figures 2I–2K).

$\alpha 6^{L9/S}$ Receptors Sensitize DA Neurons to Activation by Nicotine

To directly determine whether DA neurons in $\alpha 6^{L9/S}$ mice express hypersensitive $\alpha 6^*$ nAChRs, we prepared coronal midbrain slices (Figure 4A) and made patch-clamp recordings from VTA DA neurons in whole-cell configuration. DA neurons can be identified based on expression of tyrosine hydroxylase (TH) (Figure 4B) and with electrophysiology (Wooltorton et al., 2003); DA neurons exhibit pacemaker firing (1–5 Hz), membrane potential adaptations in response to hyperpolarizing current injections, and often express I_h . We recorded currents induced by local nicotine application (Figure 4C) at a range of concentrations. Nicotinic currents in $\alpha 6^{L9/S}$ neurons were markedly hypersensitive to nicotine; puffs of nicotine (1 μ M) elicited inward currents that were larger than those seen in WT neurons at any concentration (Figure 4D). At all nicotine concentrations, $\alpha 6^{L9/S}$ neurons of both transgenic lines showed larger average peak current responses than WT neurons (Figure 4E). Normalizing the peak current amplitude to cell capacitance yielded identical results (Figure S6).

Whole-cell responses to 1 μ M nicotine were $\geq 90\%$ blocked when α CtxMII (100 nM) was added to the perfusate (Figure 4F, upper panel). α CtxMII block persisted 30 min after washout, consistent with previous work (McIntosh et al., 2004). Likewise, responses to 1 μ M nicotine were 100% blocked in the presence of dihydro- β -erythroidine (DH β E, 2 μ M), a potent inhibitor of most

$\beta 2^*$ receptors (Figure 4F, lower panel). Although the general kinetics of these hypersensitive responses suggests that $\alpha 6^{L9/S}$ receptors are postsynaptic, a presynaptic mechanism is not excluded by the data. To address this, we inhibited AMPA/kainate receptors with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 15 μ M) and voltage-gated Na^+ channels with tetrodotoxin (TTX, 0.5 μ M). Inward current responses to 1 μ M nicotine were unaffected by either CNQX or TTX (Figure 4G). Furthermore, we found no effect ($n = 6$, 0% block) on peak current amplitude (1 μ M nicotine) in the presence of methyllycaconitine (MLA, 10 nM) (Figure 4G). Thus, hypersensitive $\alpha 6^*$ responses were consistent with a postsynaptic mechanism.

VTA DA neuron pacemaker firing was unaltered in WT versus $\alpha 6^{L9/S}$ slices (Figure 4I). To determine whether hypersensitive nicotinic receptors in $\alpha 6^{L9/S}$ neurons were capable of acutely altering the excitability of VTA DA neurons, we recorded action potential firing in response to local nicotine application. Nicotine (1 μ M) induced a transient increase in the instantaneous firing rate and a depolarization of the membrane potential (Figure 4H). There was a significant increase in the firing rate for $\alpha 6^{L9/S}$ but not WT cells (Figure 4I).

We noticed greater fluctuations in the holding current in $\alpha 6^{L9/S}$ VTA DA neurons than in WT (Figure 5A). This suggests that some $\alpha 6^{L9/S}$ channels are tonically active, reminiscent of previous observations in $\alpha 4$ nAChR $L9/S$ and $L9/A$ knockin mice (Labarca et al., 2001; Shao et al., 2008). To determine whether this change in resting membrane conductance was due to active $\alpha 6^{L9/S}$ channels, $\alpha 6^*$ receptors were blocked by application of α CtxMII. $\alpha 6$ blockade completely eliminated this increased channel noise in all $\alpha 6^{L9/S}$ cells tested (Figure 5B). WT neurons bathed in α CtxMII served as a control (Figure 5C). To quantify this, we measured the root-mean-square (RMS) value of the fluctuations in WT and $\alpha 6^{L9/S}$ voltage-clamp recordings. There was a significant decrease in the noise for $\alpha 6^{L9/S}$ cells in the presence of α CtxMII (Figure 5D). We found no effect of CNQX, picrotoxin, or TTX on the increased membrane noise in $\alpha 6^{L9/S}$ cells (Figure S7). DH β E, however, completely eliminated the increased noise similar to α CtxMII (Figure S7). These results further demonstrate that VTA DA neurons express functional, hypersensitive $\alpha 6^*$ nAChRs and that these receptors may be activated by local ACh.

We examined the properties of hypersensitive $\alpha 6^*$ receptors in DA neurons of the substantia nigra pars compacta (SNc) and found similar results to the VTA. SNc DA neurons from $\alpha 6^{L9/S}$ mice expressed hypersensitive nicotinic currents relative to WT, and firing rates were excited by 1 μ M nicotine in $\alpha 6^{L9/S}$ but not WT cells (Figures S8A and S8B). Finally, $\alpha 6^{L9/S}$ SNc DA neurons expressed tonically active, α CtxMII-sensitive $\alpha 6^*$ receptors (Figure S8C).

Selective Expression of Functional $\alpha 6^*$ Receptors in Midbrain DA Neurons

Although expression data suggest selective expression of $\alpha 6^*$ nAChRs in DA neurons, no electrophysiological experiments supporting this idea have been published. Midbrain DA neurons typically express D2-class autoreceptors, in contrast to midbrain GABAergic neurons (Nashmi et al., 2007). We recorded from 15 VTA neurons, ten of which expressed $\alpha 6^*$ nAChRs (based on large inward current responses to 1 μ M nicotine) and five which

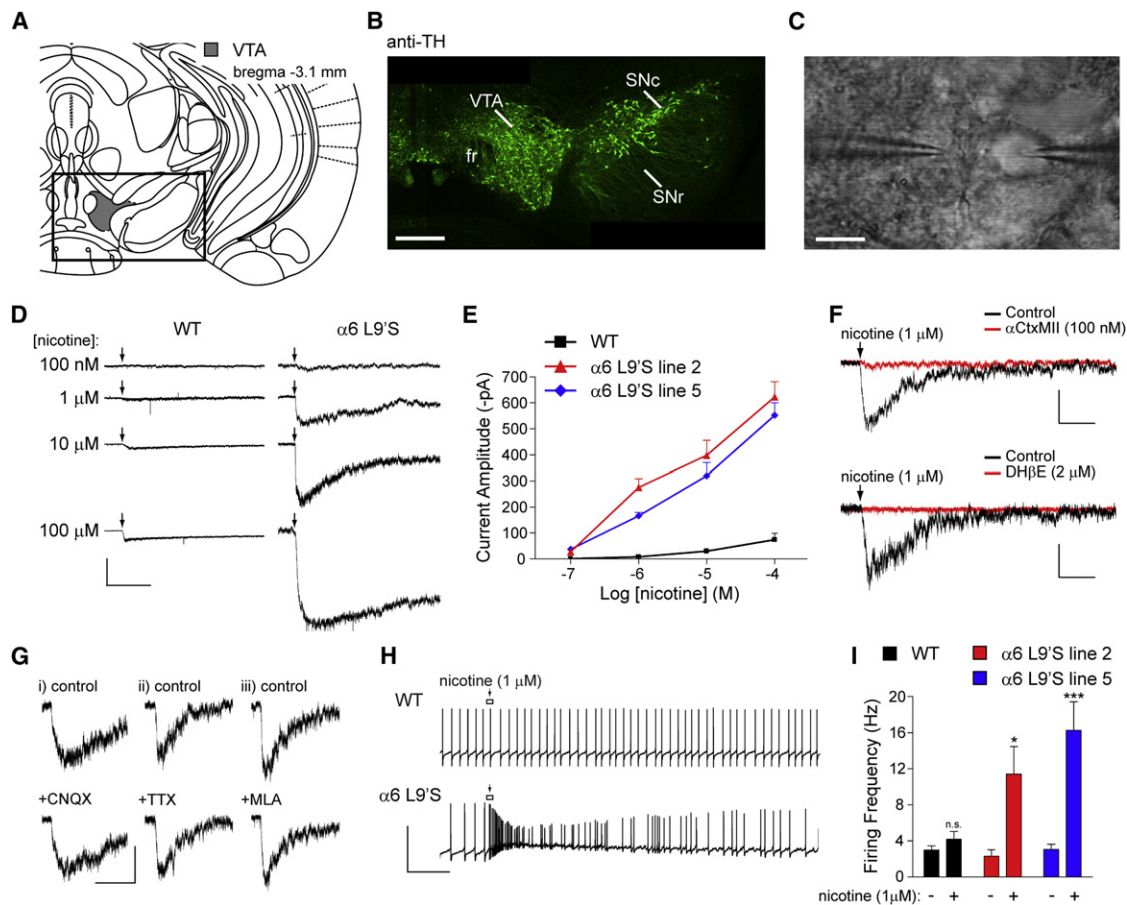


Figure 4. Ventral Tegmental Area DA Neurons Express Hypersensitive nAChRs

(A) Diagram of coronal sections from mouse brain containing ventral tegmental area (Franklin and Paxinos, 2008).

(B) Tyrosine hydroxylase (TH) staining of DA neurons in coronal slices. Coronal slices (250 μ m; bregma -3.1 mm) from an $\alpha 6^{L9'S}$ mouse were fixed and stained for TH as described in Supplemental Experimental Procedures. The boxed area from (A) is shown. Scale bar: 350 μ m.

(C) Micrograph of VTA neuron studied with local nicotine application. A VTA neuron is recorded via the patch pipette (right), and the cell body is stimulated with a nicotine-filled micropipette (left). Scale bar: 20 μ m.

(D) Hypersensitive nAChRs in VTA neurons. Patch-clamp recordings were taken from VTA DA neurons in coronal slices from $\alpha 6^{L9'S}$ and WT mice. Neurons were voltage clamped at -60 mV, and nicotine at the indicated concentration was locally applied to the cell body using a picospritzer (250 ms). Except during drug application, the micropipette tip was kept several hundred microns from the cell to prevent receptor desensitization. Scale bars: 200 pA, 5 s.

(E) Concentration-response relationship for hypersensitive nicotinic responses in WT and $\alpha 6^{L9'S}$ mouse lines. The average peak amplitude of the cellular response for each mouse line and each nicotine concentration is plotted.

(F) Hypersensitive nAChRs are inhibited by α CtxMII and dihydro- β -erythroidine (DH β E). Nicotine (1 μ M) was locally applied to VTA DA neurons in $\alpha 6^{L9'S}$ slices before (black trace) and after (red trace) 10 min bath application of α CtxMII (100 nM) or DH β E (2 μ M) ($n = 5$ for each antagonist). Scale bars: 100 pA, 3 s.

(G) Hypersensitive nAChR activation is independent of AMPA receptor function, action potentials, and $\alpha 7^+$ nAChRs. The indicated drug was bath applied to VTA DA neurons in $\alpha 6^{L9'S}$ slices, followed by activation of nAChRs with local application of 1 μ M nicotine. Peak current amplitudes in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), tetrodotoxin (TTX), or methyllycaconitine (MLA) were unchanged. Results from both mouse lines were identical in all cells tested ($n = 3$ for each drug). Scale bars: (Gi) 100 pA, 3 s; (Gii) 60 pA, 3 s; (Giii) 70 pA, 3 s.

(H) Dopamine neuron firing is augmented by a low nicotine concentration only in $\alpha 6^{L9'S}$ mice. VTA dopamine neurons were held in $I = 0$ mode, and nicotine (1 μ M) was puff applied (arrow) as in (D) following a stable recording of baseline firing. A representative firing response is shown for a neuron from each genotype. Scale bars: 100 mV, 2 s.

(I) Quantification of firing responses in WT and $\alpha 6^{L9'S}$ VTA DA neurons. Peak instantaneous firing frequency values for each nicotine (1 μ M) application (one per cell) were derived and averaged for each mouse line. Average peak values are compared with the average baseline firing rate for the same group of cells. Data are reported as mean \pm SEM. * $p < 0.05$, *** $p < 0.001$.

did not. All cells expressing $\alpha 6^+$ nAChRs were sensitive to the inhibitory properties of the D2 DA receptor agonist quinpirole (Figure 6A, panel i), indicating that these cells were likely DAergic. In contrast, most $\alpha 6^+$ -negative cells did not express D2 receptors (Figure 6A, panel ii). To more accurately determine

whether $\alpha 6^+$ receptors are functionally expressed in DA and/or GABA cells in midbrain, we recorded from substantia nigra (SN) neurons in slices from WT and $\alpha 6^{L9'S}$ mice. The spatial partitioning of DA and GABA neurons in the SN pars compacta (SNc) and pars reticulata (SNr) (Figure 6B, panel i) was combined with

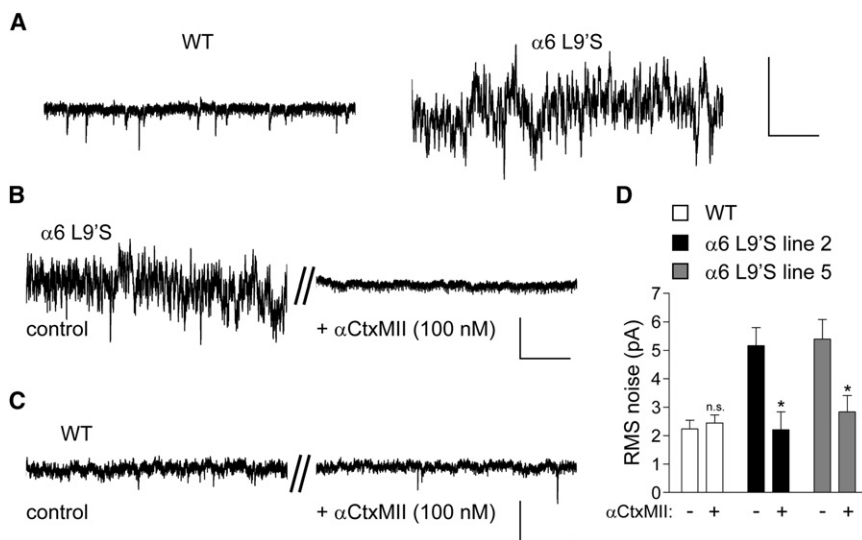


Figure 5. Spontaneous $\alpha 6$ Channel Activity in $\alpha 6^{L9/S}$ VTA DA Neurons

(A) Increased current fluctuations in voltage-clamp recordings from $\alpha 6^{L9/S}$ neurons. VTA DA neurons from WT or $\alpha 6^{L9/S}$ mice were voltage clamped at -60 mV. A segment from a representative voltage-clamp recording is shown. Scale bars: 40 pA, 0.8 s.

(B) Noise in $\alpha 6^{L9/S}$ neurons is dependent on $\alpha 6^*$ nAChRs. VTA DA neurons were voltage clamped at -60 mV, and a baseline recording was taken (control). α CtxMII was bath applied for 10 min, and a representative ($n = 4$, each line) segment of the voltage-clamp record after this 10 min period is shown (+ α CtxMII, 100 nM) for the same cell. (C) α CtxMII inhibition has no effect in WT control recordings. As a negative control for perfusion artifacts, WT neurons were assayed as in (B).

(D) Quantification of channel noise increase in $\alpha 6^{L9/S}$ mice. RMS noise values for voltage-clamp recordings from VTA DA neurons in the presence and absence of α CtxMII are shown.

Data are reported as mean \pm SEM. * $p < 0.05$. Scale bars (B and C): 20 pA, 0.5 s.

electrophysiological signatures (Figure 6B, panels ii–iv; see **Experimental Procedures**) to unambiguously identify these cell types. In whole-cell recordings from $\alpha 6^{L9/S}$ and WT SN neurons, we observed hypersensitive nicotinic responses in $\alpha 6^{L9/S}$ DA neurons, but not in GABA neurons (Figure 6C). Average peak current amplitudes were comparable between $\alpha 6^{L9/S}$ lines 2 and 5 in SNc DA neurons (Figure 6D, panel i). Average responses in WT and $\alpha 6^{L9/S}$ GABA neurons were ≤ 10 pA (Figure 6D, panel ii). Responses to nicotine at 1μ M were undetectable in WT DA and GABA neurons (Figure 6C, panel i); however, these cells responded predictably to 100μ M or 1 mM nicotine (data not shown). As a control for the specificity of our results, we recorded from SNc and SNr neurons in slices from $\alpha 4^{L9/A}$ mice. We found hypersensitive nicotinic responses in SNc and SNr neurons from these mice (Figures 6C and 6D), consistent with the idea that $\alpha 4$ is expressed in both DA and GABA cells (Nashmi et al., 2007). These results, coupled with the absence of $\alpha 6^*$ receptors in GABAergic presynaptic terminals in striatum (Figures 2I–2K), indicate that functional $\alpha 6^*$ receptors, in contrast to $\alpha 4^*$ receptors, are restricted to DA neurons in the midbrain.

The results thus far suggest increased DA tone in midbrain and/or striatum. To examine this, we studied pacemaker and nicotine-induced firing of VTA DA neurons in the absence and presence of sulpiride, a D2 DA receptor antagonist. There was no change in the ability of sulpiride to modestly increase baseline firing between WT and $\alpha 6^{L9/S}$ VTA DA cells (Figure S9). Further, sulpiride did not affect 1μ M nicotine-induced increases in firing in $\alpha 6^{L9/S}$ cells or its lack of effect in WT cells (Figure S9). To determine whether augmented striatal DA release in $\alpha 6^{L9/S}$ mice (Figure 2) could be detected in brain slices using patch-clamp recordings, we studied striatal cholinergic interneurons. These cells are readily identifiable (Figure S10A), their activity is modulated by DA, and they are the source of ACh that activates $\alpha 6^*$ receptors on presynaptic DA terminals. We detected no change in spontaneous firing between WT and $\alpha 6^{L9/S}$ cells (Figure S10B). The resting membrane potential for $\alpha 6^{L9/S}$ line 2 (but not line 5)

was hyperpolarized compared to WT (Figure S10C). Although DA may modulate I_h currents in cholinergic interneurons (Deng et al., 2007), we found no difference between WT and $\alpha 6^{L9/S}$ I_h expression or function in these cells (Figures S10D and S10E).

$\alpha 6^{L9/S}$ nAChRs in Locus Coeruleus Neurons Do Not Account for Behavioral Phenotypes in Mutant Mice

To determine whether $\alpha 6^*$ activation in $\alpha 6^{L9/S}$ mice might also stimulate locus coeruleus (LC) neuron firing, we recorded from LC neurons in coronal slices from WT and $\alpha 6^{L9/S}$ mice (Figure 7A). LC neurons express tyrosine hydroxylase (TH) (Figure 7B), exhibit spontaneous firing (1–2 Hz; Figure 7C, panel i), and lack I_h (Figure 7C, panels ii and iii). LC neurons from $\alpha 6^{L9/S}$ mice exhibited larger responses to locally applied nicotine compared to WT cells and cells from $\alpha 4^{L9/A}$ knockin mice (Figures 7D and 7E). Although these responses were sensitive to α CtxMII (Figure 7F) and therefore $\alpha 6$ dependent, they were smaller and ~ 10 -fold less sensitive to nicotine than were receptors on VTA DA neurons (compare Figures 4E and 7E). LC neurons were also able to fire action potentials in response to nicotine (Figure 7G), but at concentrations of nicotine 10-fold higher than for DA neurons (Figure 7H). The reduced sensitivity of LC $\alpha 6^*$ nAChRs relative to receptors on DA neurons suggests that they do not participate in the psychostimulant response to nicotine that we observed. In support of this, we found no change in the locomotor response to nicotine when prazosin ($\alpha 1$ AR antagonist) or yohimbine ($\alpha 2$ AR antagonist) were administered prior to challenge with nicotine (Figure 1H).

DISCUSSION

$\alpha 6^{L9/S}$ Mice Reveal a Role for $\alpha 6^*$ nAChRs in Cholinergic Modulation of DA Transmission

Here, we report several new aspects of $\alpha 6^*$ nAChR biology and its role in cholinergic regulation of DA transmission. Also, we show the behavioral effects of selectively activating DA neurons

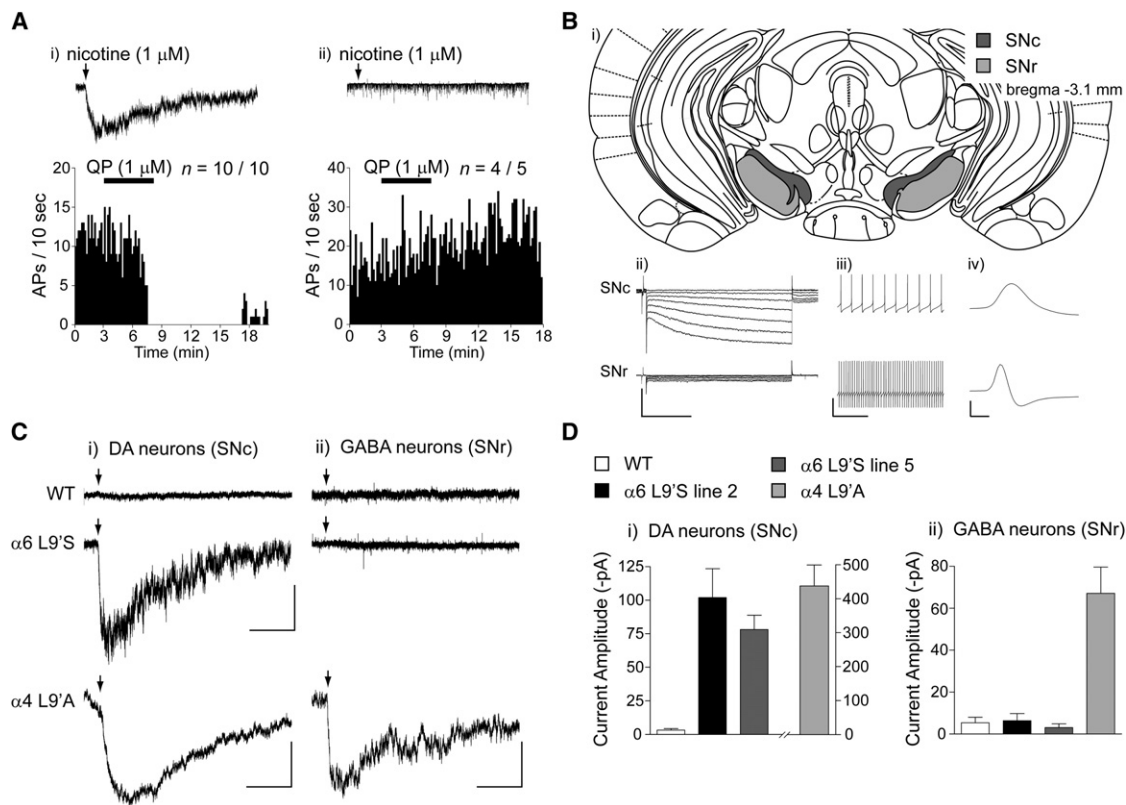


Figure 6. Functional $\alpha 6^+$ nAChRs Are Selectively Expressed in Midbrain DA, but Not GABA, Neurons

(A) VTA DA neurons expressing hypersensitive $\alpha 6^+$ nAChRs express dopamine D2-class autoreceptors. VTA DA neurons expressing $\alpha 6^{L9S}$ nAChRs were identified by the presence of large (>100 pA) inward nicotinic currents similar to Figure 4. Following nicotine application, cells were held in $I = 0$ mode, and quinpirole (QP; $1 \mu\text{M}$) was bath applied. Action potential firing in response to QP is shown for a representative neuron expressing (panel [Aii]; $n = 10/10$) or not expressing (panel [Aij]; $n = 4/5$) $\alpha 6^{L9S}$ receptors.

(B) Identification of substantia nigra (SN) DA and GABA neurons. A diagram of coronal sections (bregma -3.1 mm) from mouse brain containing SN pars compacta (SNc) and pars reticulata (SNr) is shown (Franklin and Paxinos, 2008). SN DA versus GABA neurons were identified by (Bi) location: SNc contains DA neurons whereas SNr is largely GABAergic; (Bii) DA neurons express hyperpolarization-activated cation current (I_h); (Biii) DA neurons exhibit pacemaker firing ($1\text{--}5$ Hz), whereas GABA neurons fire at >10 Hz; (Biv) DA neurons have broad spikes (>2 ms), whereas GABA neurons have narrow (≤ 1 ms) spikes. Scale bars: (Bii) 400 pA (current traces)/30 mV (voltage step), 0.5 s; (Biii) 50 mV, 1 s; (Biv) 50 mV, 1 ms.

(C) Hypersensitive $\alpha 6^+$ nAChRs reveal selective expression in DA neurons. SN DA and GABA neurons were identified according to criteria in (B). Neurons in slices from WT, $\alpha 6^{L9S}$, and $\alpha 4^{L9A}$ mice were patch clamped in whole-cell configuration, and nicotine ($1 \mu\text{M}$) was locally applied (arrow) to activate hypersensitive nAChRs. Representative recordings for each are shown. Scale bars: (WT and $\alpha 6^{L9S}$) 100 pA, 5 s; ($\alpha 4^{L9A}$) 200 pA (DA)/80 pA (GABA), 5 s.

(D) Quantification of current amplitudes from (C). Average peak current amplitude in response to $1 \mu\text{M}$ nicotine for each genotype and cell type is shown. Data are expressed as mean \pm SEM.

(Figure 1). Our electrophysiology and neurochemistry experiments reveal a major role for $\alpha 6^+$ nAChRs in regulating both DA neuron firing (Figures 4 and S8) and synaptic release of DA in the striatum (Figures 2 and 3). Our finding that $\alpha 6^+$ nAChRs are largely excluded from midbrain GABA neurons (Figure 6) and from striatal GABAergic terminals (Figures 2I–2K), in stark contrast to $\alpha 4\beta 2^+$ nAChRs, is sustained by our behavioral data suggesting relatively unrestrained DA transmission in $\alpha 6^{L9S}$ mutant mice. We propose a model in which specific functional expression of high-sensitivity $\alpha 6^{L9S}$ nAChRs in DA neurons renders these cells selectively hypersensitive (Figure S11) to activation by endogenous ACh (Figure 8B) or by exogenous nicotine (Figure 8D). This likely reflects that, in WT mice or in humans, high-affinity $\alpha 6^+$ nAChRs are specifically poised to modulate the activity of monoamine neurotransmitters such as DA. These

studies provide long-sought sufficiency data for $\alpha 6^+$ nAChRs, complementing studies utilizing $\alpha 6^+$ loss-of-function mutations and pharmacological blockade.

In Vivo $\alpha 6^+$ nAChR Stoichiometry and Biophysical Properties Revealed by $\alpha 6^{L9S}$ Mice

We observed similar levels of αCtxMII binding in $\alpha 6^{L9S}$ brains compared to WT controls (Figures S1F and S1G). This is interesting in light of the fact that the two $\alpha 6^{L9S}$ lines harbor multiple copies of the transgene (Figure S1D). Unlike an exon-replacement knockin approach, $\alpha 6^{L9S}$ BAC transgenic mice retain two copies of WT *Chrna6*. In DA neurons, $\alpha 6$ and $\alpha 4$ subunits compete for common, limiting nAChR subunits such as $\beta 2$, possibly $\beta 3$ and $\alpha 5$, and probably for unknown assembly factors or chaperone proteins that may be specific to this cell type. As a result,

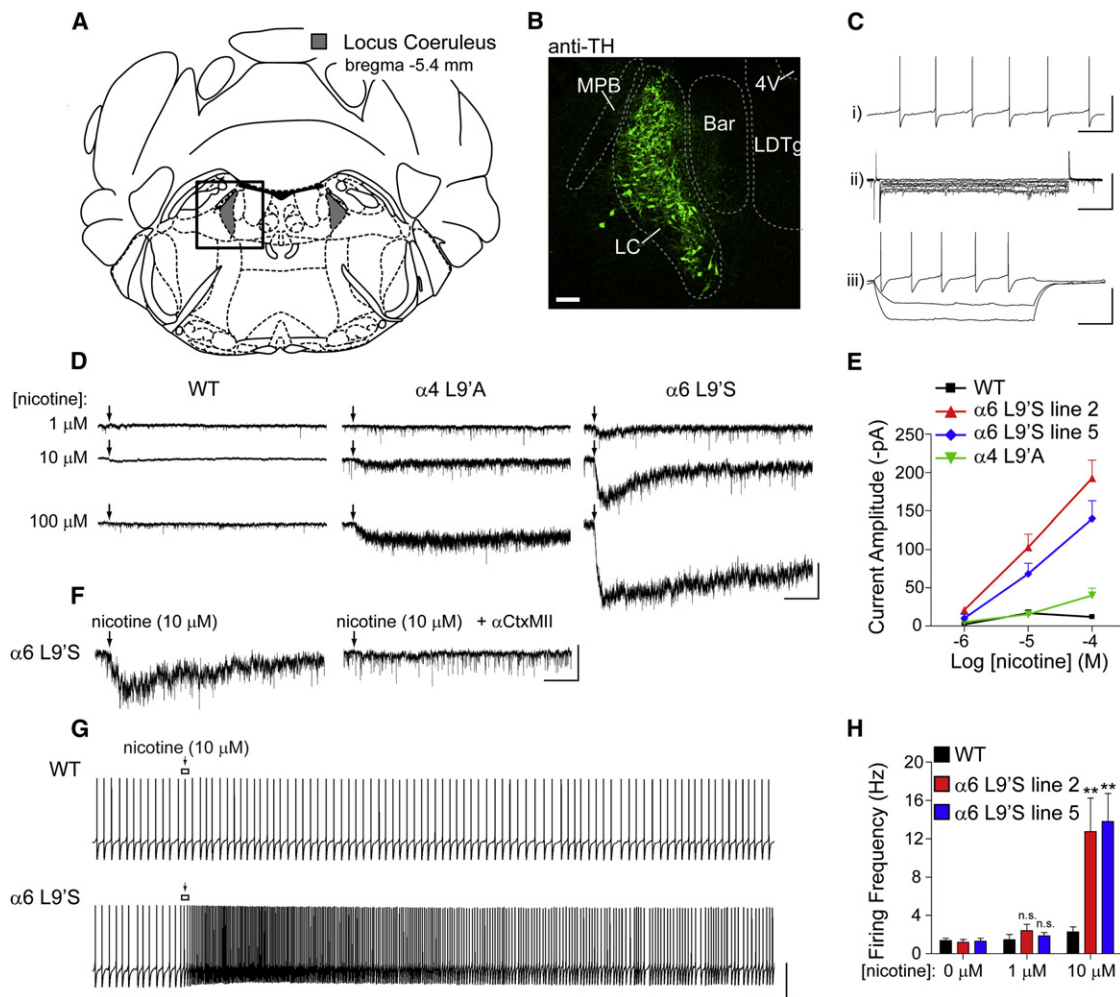


Figure 7. Modest Hypersensitivity to Nicotine at $\alpha 6^{L9S}$ nAChRs in Noradrenergic Locus Coeruleus Neurons

(A) Diagram of coronal sections (bregma -5.4 mm) containing locus coeruleus (Franklin and Paxinos, 2008).

(B) Tyrosine hydroxylase (TH) staining of NE neurons in coronal slices. Coronal slices ($200 \mu\text{m}$; bregma -5.4 mm) from an $\alpha 6^{L9S}$ mouse were fixed and stained for TH as described in Supplemental Experimental Procedures. The boxed area from (A) is shown. Scale bar: $100 \mu\text{m}$. MPB, medial parabrachial nucleus; LC, locus coeruleus; Bar, Barrington's nucleus; LDTg, laterodorsal tegmental nucleus; 4V, fourth ventricle.

(C) Electrophysiological identification of LC neurons. LC neurons are identified by (Ci) pacemaker firing at $1\text{--}2$ Hz, (Cii) lack of I_h , and (Ciii) lack of membrane potential "sag" for hyperpolarizing current pulses (responses shown for injection of -80 , -40 , and $+20$ pA). Scale bars: (Ci) 70 mV, 1.5 s; (Cii) 150 pA, 350 ms; (Ciii) 70 mV, 500 ms.

(D) Hypersensitive nicotinic responses in LC neurons. Patch-clamp recordings were taken from LC neurons in coronal slices from WT, $\alpha 4^{L9A}$, and $\alpha 6^{L9S}$ mice. Neurons were voltage clamped at -60 mV, and nicotine at the indicated concentration was locally applied (arrow) to the cell body (250 ms).

(E) Concentration-response relationship for hypersensitive nicotinic responses in WT, $\alpha 4^{L9A}$, and $\alpha 6^{L9S}$ mice. The average peak amplitude of the cellular response for each mouse line and each nicotine concentration is plotted.

(F) Hypersensitive $\alpha 6^{L9S}$ nAChRs are inhibited by αCtXMII . Nicotine ($10 \mu\text{M}$) was locally applied (arrow) to LC neurons in $\alpha 6^{L9S}$ slices before and after 10 min bath application of αCtXMII (100 nM) ($n = 3$). Scale bars: 50 pA, 4 s.

(G) Noradrenergic neuron firing is augmented by a moderate nicotine concentration only in $\alpha 6^{L9S}$ mice. Following a stable recording of baseline action potential firing, nicotine ($10 \mu\text{M}$) was puff applied (arrow) as in (D). Exemplar responses are shown for a WT and $\alpha 6^{L9S}$ neuron. Scale bars: 70 mV, 3 s.

(H) Quantification of firing responses in WT and $\alpha 6^{L9S}$ LC neurons. Peak instantaneous firing frequency values for each nicotine concentration were derived and averaged. Average peak values are compared with the average baseline firing rate ($0 \mu\text{M}$ nicotine) for the same group of cells.

Data are reported as mean \pm SEM. $^{**}p < 0.01$.

the level of functional $\alpha 6^*$ expression in $\alpha 6^{L9S}$ neurons is determined by a competition between WT and L9/S $\alpha 6$ subunits. Indeed, for every agonist tested, we observed diminished peak αCtXMII -resistant ($\alpha 4\beta 2^*$ dependent) DA release in $\alpha 6^{L9S}$ ST/OT (Figures 2 and 3) despite equal levels of $\alpha 4\beta 2^*$ -binding sites

(Figure S1G). Future crosses of $\alpha 6^{L9S}$ mice to $\alpha 4$, $\beta 2$, and $\beta 3$ nAChR heterozygous or homozygous KO mice will yield further insights into nAChR subunit stoichiometry in vivo.

We noted a large increase in the potency and efficacy of nicotine in whole-cell recordings from $\alpha 6^{L9S}$ DA neurons (Figures 4D

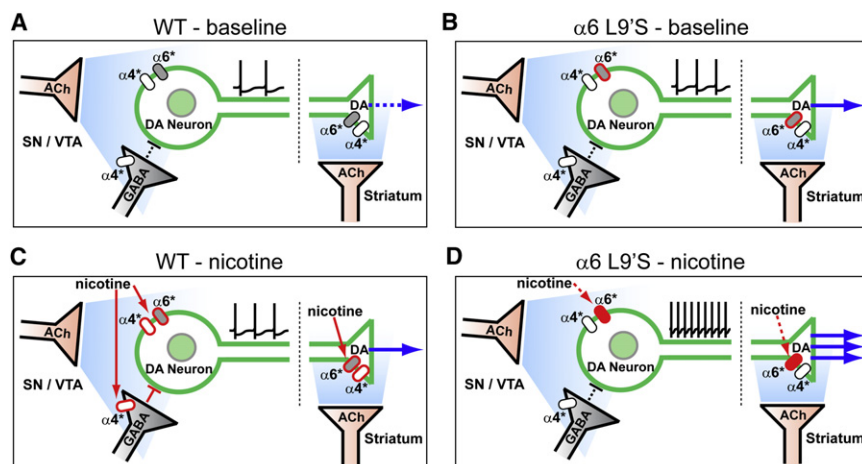


Figure 8. Hypersensitive DA Neurons Alter Basal Ganglia Circuitry for Cholinergic Transmission and Responses to Nicotine

(A–D) Schematic for baseline and nicotine-induced activation of DA neurons. In WT mice, background (A) ACh weakly stimulates somatic and presynaptic $\alpha 4^*$ and $\alpha 6^*$ nAChRs, leading to tonic DA release in ST. In $\alpha 6^{L9'S}$ mice, baseline ACh (B) activation of $\alpha 6^*$ nAChRs may cause heightened DA release via increased somatic and presynaptic nAChR activity. In WT (C), systemic nicotine (≥ 0.5 mg/kg, i.p.) activates $\alpha 4^*$ and $\alpha 6^*$ nAChRs on DA neurons, and $\alpha 4^*$ nAChRs on GABA neurons/terminals in midbrain. This, coupled with activation of presynaptic $\alpha 4^*$ and $\alpha 6^*$ nAChRs, increases DA release in ST. In $\alpha 6^{L9'S}$ mice (D), systemic nicotine (≤ 0.15 mg/kg, i.p.) specifically activates somatic and presynaptic $\alpha 6^*$ nAChRs on DA neurons without activating $\alpha 4^*$ nAChRs on inhibitory GABAergic inputs, which strongly stimulates DA release.

and 4E). With respect to nicotinic channel engineering, the present studies are analogous to electrophysiological and Ca^{2+} flux-based measurements of hypersensitive nicotinic responses in $\alpha 4^{L9'S}$, $\alpha 4^{L9'A}$, and $\alpha 7^{L9'T}$ knockin mice (Fonck et al., 2005; Labarca et al., 2001; Tapper et al., 2004; Wooltorton et al., 2003). The augmented responses (increased efficacy) to agonist likely reflect an increased maximal probability of channel opening, P_{open} , conferred by the L9'S mutation (Labarca et al., 1995), while the increased potency results from this mutation shifting the agonist concentration-response relation to lower concentrations (Revah et al., 1991). The increased noise in voltage-clamp recordings from $\alpha 6^{L9'S}$ neurons probably arises from one of two effects: (1) the presence of ACh in the slice preparation that is secreted from terminals of severed mesopontine cholinergic axons or (2) unliganded openings. Future in vivo studies, such as recordings from awake, behaving animals, will help confirm whether $\alpha 6^{L9'S}$ mouse spontaneous behavioral phenotypes are caused by the former or the latter.

Contrasting Cell-Specific Expression of $\alpha 4^*$ versus $\alpha 6^*$ nAChRs Leads to Behavioral Differences in $\alpha 4$ and $\alpha 6$ Gain-of-Function Mutant Mice

$\alpha 6^{L9'S}$ mice are viable and fertile, whereas full expression of $\alpha 4^{L9'S}$ receptors causes neonatal lethality due to excitotoxic death of DA neurons (Labarca et al., 2001). This could be due to differential expression of $\alpha 4$ and $\alpha 6$ in development; unlike $\alpha 4$ expression, peak $\alpha 6$ expression occurs well after birth (Azam et al., 2007). It is also possible that $\alpha 6^{L9'S}$ receptors are comparatively insensitive to activation by choline at concentrations found in CSF.

In the mesostriatal and mesolimbic DA system, $\alpha 4\beta 2^*$ nAChRs are expressed in DA neuron cell bodies, dendrites, and axon terminals, as well as in cell bodies and axon terminals of midbrain and striatal GABAergic neurons. Conclusive evidence for $\alpha 6^*$ nAChR expression, however, is restricted to DA neuron cell bodies and axon terminals. Our present results show that, in midbrain, manipulating $\alpha 6^*$ nAChR sensitivity affects only DA neurons (Figures S11, 8B, and 8D), whereas sensitized $\alpha 4^*$ receptors simultaneously increase the sensitivity of DA neurons

and their inhibitory GABAergic inputs (Figures 6, 8A, and 8C). This circuit-level difference explains a distinction between the locomotor effects of nicotine in the two gain-of-function mouse strains. WT mice display a hypolocomotor response to nicotine, and $\alpha 4^{L9'A}$ mice recapitulate the WT response, only at much lower doses (Tapper et al., 2007). On the other hand, $\alpha 6^{L9'S}$ mice exhibit a sign change: psychomotor stimulation by nicotine (Figure 1F).

This cell-type difference in expression between $\alpha 4$ and $\alpha 6$ nAChR subunits may also lead to the behavioral differences between $\alpha 4^{L9'A}$ and $\alpha 6^{L9'S}$ mice in response to repeated nicotine injections. Repeated, selective activation of $\alpha 4^*$ nAChRs produces locomotor sensitization (Tapper et al., 2004, 2007), whereas repeated activation of $\alpha 6^*$ nAChRs produces neither tolerance nor sensitization (Figure 1I). Locomotor sensitization may require nicotinic activation of GABAergic transmission, which is afforded by $\alpha 4^*$ nAChRs but not $\alpha 6^*$ nAChRs (Nashmi et al., 2007). Alternatively, the mechanism of sensitization could involve nAChR upregulation, to which $\alpha 4\beta 2^*$ receptors are particularly prone (Nguyen et al., 2003) but to which $\alpha 6^*$ receptors are apparently resistant (Perry et al., 2007).

$\alpha 6^*$ nAChRs Preferentially Modulate the Mesolimbic versus Mesostriatal DA System

The VTA and NAc (mesolimbic DA pathway) are key mediators of the addictive properties of nicotine (Corrigall et al., 1994), and a recent report using pharmacological blockade suggests that $\alpha 6^*$ nAChRs specifically mediate cholinergic modulation of DA release in NAc (Exley et al., 2007). Our results support this in two ways: (1) we observe greater expression of $\alpha 6^*$ currents in VTA versus SNc neurons (compare Figures 4E, 6D, and S8A), and (2) DA release is more strongly controlled by $\alpha 6^*$ on VTA-derived terminals versus SNc-derived terminals (compare EC_{50} values in Figures 2G and 2H). Although we cannot completely distinguish the contribution of dorsal versus ventral striatum in our behavioral experiments, our DA release data suggest that the first $\alpha 6^*$ nAChRs significantly activated by nicotine are those in the mesolimbic pathway.

VTA $\beta 2^*$ nAChRs are critical mediators of exploratory behavior in mice (Granon et al., 2003; Maskos et al., 2005). The VTA is

important for curiosity or the response to novelty, perhaps by responding to cholinergic excitation to mediate the switch to burst firing in DA neurons. If VTA $\beta 2^*$ nAChRs are necessary for normal responses to novelty, then it is not surprising that sensitized VTA $\beta 2^*$ nAChRs such as $\alpha 6^{L9/S}\beta 2^*$ in mutant mice render the animals hypersensitive to novelty (Figures 1C and 1D). This phenotype is much smaller or absent in $\alpha 4^{L9/A}$ mice, reflecting the relative importance of selectively activating DA in eliciting this response.

$\alpha 6^{L9/S}$ Mice Reveal a Role for Cholinergic Transmission in Behavioral Hyperactivity

In midbrain, ACh released from mesopontine cholinergic terminals acts on DA neuron nAChRs in vivo (Mameli-Engvall et al., 2006) (Figure 8A). $\alpha 6^*$ nAChRs have the highest known sensitivity to ACh, making them excellent candidates to mediate the stimulatory action of endogenous ACh on DA neurons. The tonic activation of $\alpha 6^*$ nAChRs that we observe in midbrain slices is likely due to endogenous ACh. Although we observe no difference in DA neuron baseline firing in vitro (Figures 4I and S8B), tonic midbrain $\alpha 6^*$ nAChR activation may be sufficient in vivo to contribute to behavioral phenotypes in $\alpha 6^{L9/S}$ mice. In striatum, tonic extracellular DA is controlled by presynaptic nAChRs via continuous, low-level ACh released from cholinergic interneurons (Zhou et al., 2001). This acts to maintain a high probability of DA release from the terminal during tonic firing (Rice and Cragg, 2004; Zhang and Sulzer, 2004). Sensitization of $\alpha 6^*$ nAChRs (in $\alpha 6^{L9/S}$ mice) likely modifies DA release at presynaptic terminals in addition to effects at DA neuron cell bodies; DA terminals with $\alpha 6^{L9/S}$ channels presumably have a decreased failure rate for single spike-induced release. This is supported by in vitro electrochemical studies of $\alpha 6^*$ -dependent DA release (Exley et al., 2007). Thus, genetic sensitization of DA neurons to ACh in midbrain and facilitation of DA release in striatum (Figure 8B) could easily account for the home cage hyperactivity and the sustained hyperactivity of $\alpha 6^{L9/S}$ mutant mice when placed in a novel environment. These phenotypes are reminiscent of DA transporter knockdown mice, which also show hyperactivity and impaired response habituation (Zhuang et al., 2001). Although $\alpha 6^*$ -regulated norepinephrine (NE) release does not contribute to nicotine psychomotor activation (Figure 1H), our results do not rule out a role for NE in $\alpha 6^{L9/S}$ spontaneous hyperactivity. Future studies on the mechanisms of $\alpha 6^{L9/S}$ mouse hyperactivity could be useful in understanding the causes of human hyperactivity, such as that observed in ADHD.

In $\alpha 6^{L9/S}$ mice, low-dose nicotine stimulates psychomotor activation similar to amphetamine in WT animals (Figure 1E). Nicotine thus recapitulates the spontaneous hyperactivity we observe, though with different kinetics. The difference in response magnitude and duration between responses to novelty and responses to nicotine may reflect different agonist concentration and desensitization kinetics. After ACh is released from nerve terminals, it is hydrolyzed by acetylcholinesterase (AChE), which has a turnover rate of $10^4/s$ (Wathey et al., 1979) and is abundant in DAergic areas. ACh may not reach concentrations, or remain long enough, to significantly desensitize $\alpha 6^*$ nAChRs, perhaps even those with mutant L9/S subunits. In contrast, nicotine is eliminated with a half-life of ~ 7 min (Matta et al., 2007)—nearly 10^7 times longer—and can therefore desensitize receptors, es-

pecially $\beta 2^*$ nAChRs (Mansvelder et al., 2002). Bolus injections of nicotine in $\alpha 6^{L9/S}$ mice potentially activate mutant receptors, and the locomotor response decay kinetics could therefore be dominated by both receptor desensitization and metabolic breakdown of nicotine.

Therapeutic Targeting of DA Disorders with $\alpha 6^*$ nAChRs

The cholinergic system is targeted by several drugs used to treat neural disorders such as Alzheimer's disease and Parkinson's disease (PD). There is a well-documented inverse correlation between smoking and PD (Ritz et al., 2007), and other disorders that can be treated with DA drugs (ADHD, schizophrenia) are associated with a high incidence of smoking (de Leon et al., 1995; Pomerleau et al., 1995). These findings suggest the important role played by the cholinergic system in DA transmission. Our results show that a sensitized response in DA neurons to endogenous ACh may cause a behaviorally relevant state of excess DA. Manipulations to decrease $\alpha 6^*$ nAChR function may, therefore, be a useful treatment for human disorders involving excess DA. This could be in the form of a competitive antagonist or via viral gene therapy designed to eliminate $\alpha 6^*$ activity. On the other hand, patients with PD (low DA) may be aided by $\alpha 6^*$ agonists or allosteric modulators to augment DA release from residual DA terminals (Quirk and McIntosh, 2006). Our data clearly show that an $\alpha 6$ -selective compound potentially stimulates both DA release and locomotor activity (Figure 3). Further, the absence of sensitization or tolerance to repeated $\alpha 6^*$ activation (Figure 1I) suggests that clinical $\alpha 6^*$ agonists may have reduced abuse liability. Unlike L-DOPA or direct DA receptor agonists/antagonists, compounds manipulating DA neuron firing by targeting $\alpha 6^*$ nAChRs might avoid well known use-dependent side effects such as dyskinesias.

EXPERIMENTAL PROCEDURES

Bacterial Artificial Chromosome Recombineering and Transgenesis

For a complete description of the methods used for recombineering and transgenesis, see Supplemental Data. A bacterial artificial chromosome (BAC), RP24-149112, containing the mouse $\alpha 6$ nicotinic receptor subunit gene (*Chrna6*) was obtained from the BACPAC Resource Center (BPRC) at Children's Hospital Oakland Research Institute (Oakland, CA). BAC recombineering was carried out using a Counter Selection BAC modification kit (GeneBridges; Heidelberg, Germany). $\alpha 6$ nAChR Leucine 280 (Leu9') was mutated to a serine using a two-step selection/counterselection protocol. First, a 15 bp stretch of *Chrna6* exon 5 containing the coding sequence for V278 through L282 was replaced with a cassette containing a tandem selection (neo)/counterselection (rpsL) marker. Neo was used to select positive recombinants, and an engineered NotI restriction site pair flanking the selection cassette was used to confirm the location of the exogenously inserted DNA within the BAC. In the second step, *Chrna6* exon 5 was restored using counterselection. Bacterial cells were placed under selective pressure (via streptomycin sensitivity gained by insertion of the rpsL marker) to lose the neo-rpsL cassette and replace it with nonselectable DNA engineered to insert the Leu9' to Ser (L280S) mutation. The resultant strain harbored a BAC with no ectopic DNA in or around *Chrna6*.

Injection-grade $\alpha 6^{L9/S}$ BAC DNA was prepared via double CsCl banding (Lofstrand Labs; Gaithersburg, MD). To produce transgenic animals, BAC DNA was injected into the male pronucleus of recently fertilized FVB/N embryos and implanted into pseudopregnant Swiss-Webster surrogates. Transgenic founders were identified using tail biopsy DNA and PCR primers designed to detect the L9/S mutation. Founders were crossed to C57BL/6J

(Jackson Labs; Bar Harbor, ME) to obtain germline transmission and to establish a colony, and transgenic mice were continually backcrossed to C57BL/6J.

Locomotor Activity

Locomotor activity was assayed similar to Fonck et al. (2005) and is described in Supplemental Data.

Neurotransmitter Release from Striatal Synaptosomes

DA and GABA release from striatal synaptosomes was carried out similar to Grady et al. (2002) and Nashmi et al. (2007) and is described in Supplemental Data.

Patch-Clamp Electrophysiology

Methods for patch-clamp recordings are completely detailed in Supplemental Data. Transgenic and nontransgenic mice (midbrain, P17–P25; locus coeruleus, P21–P28; striatum, P42–P56) were anesthetized, followed by cardiac perfusion with oxygenated (95% O₂/5% CO₂) ice-cold glycerol-based artificial CSF (gACSF). Coronal slices (midbrain, striatum: 250 μ m; pons: 200 μ m) were cut and allowed to recover for at least 1 hr at 32°C in regular, oxygenated artificial CSF (ACSF). Somatic recordings from visually identified midbrain, pontine, or striatal neurons were obtained using patch electrodes filled with internal pipette solution containing 135 mM potassium gluconate, 5 mM EGTA, 0.5 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM GTP. Recordings were taken at 32°C with an Axopatch 1D amplifier, a 16 bit Digi-data 1322A A/D converter, and pCLAMP9.2 software (all Molecular Devices Axon; Sunnyvale, CA). For nicotine application, a drug-filled micropipette was used to locally apply nicotine using pressure application. A piezoelectric translator rapidly moved the nicotine-filled pipette close to and away from the cell to avoid nAChR desensitization.

Immunohistochemistry and Spectral Confocal Imaging

Coronal brain slices cut for patch-clamp recording were processed similar to Lerchner et al. (2007) and stained according to Drenan et al. (2005). Spectral confocal imaging was similar to Drenan et al. (2008). See Supplemental Data for more details.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Figures, and Tables and can be found with this article online at [http://www.neuron.org/supplemental/S0896-6273\(08\)00756-3](http://www.neuron.org/supplemental/S0896-6273(08)00756-3).

ACKNOWLEDGMENTS

We thank members of the Lester lab for helpful discussions. Special thanks to B. Drenan. Thanks to C. Wageman, E. Myers, C. Xiao, A. Tapper, R. Nashmi, C. Fonck, J. Schwarz, J. Jankowsky, M. Liu, P. Deshpande, S. Benazouz, and C. Zhou. This work was supported by H.H.M.I. (N.H., J.M. Miwa, S.B.) and grants from NIH (DA09121, DA17279, and NS11756 to H.A.L.; DA19375 to H.A.L. and M.J.M.; DA03194 to M.J.M.; DA12242 to M.J.M. and P.W.; MH53631 and GM48677 to J.M. McIntosh), the Moore Foundation, the Croll Research Foundation (to J.M. Miwa) and the California Tobacco Related Disease Research Program (TRDRP; 12RT-0245 to H.A.L.). R.M.D. was supported by postdoctoral fellowships from TRDRP (15FT-0030) and NIH (DA021492 and NS007251).

Accepted: September 4, 2008

Published: October 8, 2008

REFERENCES

Azam, L., and McIntosh, J.M. (2006). Characterization of nicotinic acetylcholine receptors that modulate nicotine-evoked [³H]norepinephrine release from mouse hippocampal synaptosomes. *Mol. Pharmacol.* 70, 967–976.

Azam, L., Winzer-Serhan, U.H., Chen, Y., and Leslie, F.M. (2002). Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs within midbrain dopamine neurons. *J. Comp. Neurol.* 444, 260–274.

Azam, L., Chen, Y., and Leslie, F.M. (2007). Developmental regulation of nicotinic acetylcholine receptors within midbrain dopamine neurons. *Neuroscience* 144, 1347–1360.

Bencherif, M., Lovette, M.E., Fowler, K.W., Arrington, S., Reeves, L., Caldwell, W.S., and Lippiello, P.M. (1996). RJR-2403: a nicotinic agonist with CNS selectivity I. In vitro characterization. *J. Pharmacol. Exp. Ther.* 279, 1413–1421.

Bhatti, B.S., Strachan, J.P., Breining, S.R., Miller, C.H., Tahiri, P., Crooks, P.A., Deo, N., Day, C.S., and Caldwell, W.S. (2008). Synthesis of 2-(pyridin-3-yl)-1-azabicyclo[3.2.2]nonane, 2-(pyridin-3-yl)-1-azabicyclo[2.2.2]octane, and 2-(pyridin-3-yl)-1-azabicyclo[3.2.1]octane, a class of potent nicotinic acetylcholine receptor-ligands. *J. Org. Chem.* 73, 3497–3507.

Calabresi, P., Lacey, M.G., and North, R.A. (1989). Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br. J. Pharmacol.* 98, 135–140.

Cartier, G.E., Yoshikami, D., Gray, W.R., Luo, S., Olivera, B.M., and McIntosh, J.M. (1996). A new alpha-conotoxin which targets $\alpha 3\beta 2$ nicotinic acetylcholine receptors. *J. Biol. Chem.* 271, 7522–7528.

Champtiaux, N., Han, Z.Y., Bessis, A., Rossi, F.M., Zoli, M., Marubio, L., McIntosh, J.M., and Changeux, J.P. (2002). Distribution and pharmacology of $\alpha 6$ -containing nicotinic acetylcholine receptors analyzed with mutant mice. *J. Neurosci.* 22, 1208–1217.

Champtiaux, N., Gotti, C., Cordero-Erausquin, M., David, D.J., Przybylski, C., Lena, C., Clementi, F., Moretti, M., Rossi, F.M., Le Novere, N., et al. (2003). Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J. Neurosci.* 23, 7820–7829.

Corrigall, W.A., Coen, K.M., and Adamson, K.L. (1994). Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Res.* 653, 278–284.

Cui, C., Booker, T.K., Allen, R.S., Grady, S.R., Whiteaker, P., Marks, M.J., Salminen, O., Tritto, T., Butt, C.M., Allen, W.R., et al. (2003). The $\beta 3$ nicotinic receptor subunit: a component of α -Conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J. Neurosci.* 23, 11045–11053.

de Leon, J., Dadvand, M., Canuso, C., White, A.O., Stanilla, J.K., and Simpson, G.M. (1995). Schizophrenia and smoking: an epidemiological survey in a state hospital. *Am. J. Psychiatry* 152, 453–455.

Deng, P., Zhang, Y., and Xu, Z.C. (2007). Involvement of I_h in dopamine modulation of tonic firing in striatal cholinergic interneurons. *J. Neurosci.* 27, 3148–3156.

Drenan, R.M., Doupnik, C.A., Boyle, M.P., Muglia, L.J., Huettner, J.E., Linder, M.E., and Blumer, K.J. (2005). Palmitoylation regulates plasma membrane-nuclear shuttling of R7BP, a novel membrane anchor for the RGS7 family. *J. Cell Biol.* 169, 623–633.

Drenan, R.M., Nashmi, R., Imoukhuede, P., Just, H., McKinney, S., and Lester, H.A. (2008). Subcellular trafficking, pentameric assembly, and subunit stoichiometry of neuronal nicotinic acetylcholine receptors containing fluorescently labeled $\alpha 6$ and $\beta 3$ subunits. *Mol. Pharmacol.* 73, 27–41.

Exley, R., Clements, M.A., Hartung, H., McIntosh, J.M., and Cragg, S.J. (2007). $\alpha 6$ -containing nicotinic acetylcholine receptors dominate the nicotinic control of dopamine neurotransmission in nucleus accumbens. *Neuropsychopharmacology* 33, 2158–2166.

Fonck, C., Cohen, B.N., Nashmi, R., Whiteaker, P., Wagenaar, D.A., Rodrigues-Pinguet, N., Deshpande, P., McKinney, S., Kwoh, S., Munoz, J., et al. (2005). Novel seizure phenotype and sleep disruptions in knock-in mice with hypersensitive $\alpha 4^+$ nicotinic receptors. *J. Neurosci.* 25, 11396–11411.

Franklin, K.B.J., and Paxinos, G. (2008). The Mouse Brain in Stereotaxic Coordinates, Third Edition (New York, NY: Elsevier, Inc.).

Gotti, C., Moretti, M., Clementi, F., Riganti, L., McIntosh, J.M., Collins, A.C., Marks, M.J., and Whiteaker, P. (2005a). Expression of nigrostriatal $\alpha 6$ -containing nicotinic acetylcholine receptors is selectively reduced, but not eliminated, by $\beta 3$ subunit gene deletion. *Mol. Pharmacol.* 67, 2007–2015.

Gotti, C., Moretti, M., Zanardi, A., Gaimarri, A., Champtiaux, N., Changeux, J.P., Whiteaker, P., Marks, M.J., Clementi, F., and Zoli, M. (2005b). Heterogeneity and selective targeting of neuronal nicotinic acetylcholine receptor (nAChR)

- subtypes expressed on retinal afferents of the superior colliculus and lateral geniculate nucleus: identification of a new native nAChR subtype $\alpha 3\beta 2$ ($\alpha 5$ or $\beta 3$) enriched in retinocollicular afferents. *Mol. Pharmacol.* 68, 1162–1171.
- Grady, S.R., Murphy, K.L., Cao, J., Marks, M.J., McIntosh, J.M., and Collins, A.C. (2002). Characterization of nicotinic agonist-induced [3 H]dopamine release from synaptosomes prepared from four mouse brain regions. *J. Pharmacol. Exp. Ther.* 301, 651–660.
- Granon, S., Faure, P., and Changeux, J.P. (2003). Executive and social behaviors under nicotinic receptor regulation. *Proc. Natl. Acad. Sci. USA* 100, 9596–9601.
- Grenhoff, J., Aston-Jones, G., and Svensson, T.H. (1986). Nicotinic effects on the firing pattern of midbrain dopamine neurons. *Acta Physiol. Scand.* 128, 351–358.
- Kaiser, S.A., Soliakov, L., Harvey, S.C., Luetje, C.W., and Wonnacott, S. (1998). Differential inhibition by α -conotoxin-MII of the nicotinic stimulation of [3 H]dopamine release from rat striatal synaptosomes and slices. *J. Neurochem.* 70, 1069–1076.
- Kulak, J.M., Nguyen, T.A., Olivera, B.M., and McIntosh, J.M. (1997). α -conotoxin MII blocks nicotine-stimulated dopamine release in rat striatal synaptosomes. *J. Neurosci.* 17, 5263–5270.
- Labarca, C., Nowak, M.W., Zhang, H., Tang, L., Deshpande, P., and Lester, H.A. (1995). Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature* 376, 514–516.
- Labarca, C., Schwarz, J., Deshpande, P., Schwarz, S., Nowak, M.W., Fonck, C., Nashmi, R., Kofuji, P., Dang, H., Shi, W., et al. (2001). Point mutant mice with hypersensitive $\alpha 4$ nicotinic receptors show dopaminergic deficits and increased anxiety. *Proc. Natl. Acad. Sci. USA* 98, 2786–2791.
- Lanca, A.J., Adamson, K.L., Coen, K.M., Chow, B.L., and Corrigan, W.A. (2000). The pedunculo-pontine tegmental nucleus and the role of cholinergic neurons in nicotine self-administration in the rat: a correlative neuroanatomical and behavioral study. *Neuroscience* 96, 735–742.
- Le Novère, N., Zoli, M., and Changeux, J.P. (1996). Neuronal nicotinic receptor $\alpha 6$ subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *Eur. J. Neurosci.* 8, 2428–2439.
- Lerchner, W., Xiao, C., Nashmi, R., Slimko, E.M., van Trigt, L., Lester, H.A., and Anderson, D.J. (2007). Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl^- channel. *Neuron* 54, 35–49.
- Lippiello, P.M., Bencherif, M., Gray, J.A., Peters, S., Grigoryan, G., Hodges, H., and Collins, A.C. (1996). RJR-2403: a nicotinic agonist with CNS selectivity II. In vivo characterization. *J. Pharmacol. Exp. Ther.* 279, 1422–1429.
- Lu, Y., Grady, S., Marks, M.J., Picciotto, M., Changeux, J.P., and Collins, A.C. (1998). Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. *J. Pharmacol. Exp. Ther.* 287, 648–657.
- Mameli-Engvall, M., Evrard, A., Pons, S., Maskos, U., Svensson, T.H., Changeux, J.P., and Faure, P. (2006). Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. *Neuron* 50, 911–921.
- Mansvelder, H.D., and McGehee, D.S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* 27, 349–357.
- Mansvelder, H.D., Keath, J.R., and McGehee, D.S. (2002). Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* 33, 905–919.
- Maskos, U., Molles, B.E., Pons, S., Besson, M., Guiard, B.P., Guilloux, J.P., Evrard, A., Cazala, P., Cormier, A., Mameli-Engvall, M., et al. (2005). Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature* 436, 103–107.
- Matta, S.G., Balfour, D.J., Benowitz, N.L., Boyd, R.T., Buccafusco, J.J., Caggiola, A.R., Craig, C.R., Collins, A.C., Damaj, M.I., Donny, E.C., et al. (2007). Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl.)* 190, 269–319.
- McIntosh, J.M., Azam, L., Staheli, S., Dowell, C., Lindstrom, J.M., Kuryatov, A., Garrett, J.E., Marks, M.J., and Whiteaker, P. (2004). Analogs of α -Conotoxin MII are selective for $\alpha 6$ -containing nicotinic acetylcholine receptors. *Mol. Pharmacol.* 65, 944–952.
- Nashmi, R., Xiao, C., Deshpande, P., McKinney, S., Grady, S.R., Whiteaker, P., Huang, Q., McClure-Begley, T., Lindstrom, J.M., Labarca, C., et al. (2007). Chronic nicotine cell specifically upregulates functional $\alpha 4^*$ nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *J. Neurosci.* 27, 8202–8218.
- Nguyen, H.N., Rasmussen, B.A., and Perry, D.C. (2003). Subtype-selective up-regulation by chronic nicotine of high-affinity nicotinic receptors in rat brain demonstrated by receptor autoradiography. *J. Pharmacol. Exp. Ther.* 307, 1090–1097.
- Perry, D.C., Mao, D., Gold, A.B., McIntosh, J.M., Pezzullo, J.C., and Kellar, K.J. (2007). Chronic nicotine differentially regulates $\alpha 6$ - and $\beta 3$ -containing nicotinic cholinergic receptors in rat brain. *J. Pharmacol. Exp. Ther.* 322, 306–315.
- Pomerleau, O.F., Downey, K.K., Stelson, F.W., and Pomerleau, C.S. (1995). Cigarette smoking in adult patients diagnosed with attention deficit hyperactivity disorder. *J. Subst. Abuse* 7, 373–378.
- Quik, M., and McIntosh, J.M. (2006). Striatal $\alpha 6^*$ nicotinic acetylcholine receptors: potential targets for Parkinson's disease therapy. *J. Pharmacol. Exp. Ther.* 316, 481–489.
- Revah, F., Bertrand, D., Galzi, J.L., Devillers-Thiery, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M., and Changeux, J.P. (1991). Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. *Nature* 353, 846–849.
- Rice, M.E., and Cragg, S.J. (2004). Nicotine amplifies reward-related dopamine signals in striatum. *Nat. Neurosci.* 7, 583–584.
- Ritz, B., Ascherio, A., Checkoway, H., Marder, K.S., Nelson, L.M., Rocca, W.A., Ross, G.W., Strickland, D., Van Den Eeden, S.K., and Gorell, J. (2007). Pooled analysis of tobacco use and risk of Parkinson disease. *Arch. Neurol.* 64, 990–997.
- Salminen, O., Murphy, K.L., McIntosh, J.M., Drago, J., Marks, M.J., Collins, A.C., and Grady, S.R. (2004). Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol. Pharmacol.* 65, 1526–1535.
- Schultz, W. (2002). Getting formal with dopamine and reward. *Neuron* 36, 241–263.
- Shao, X.M., Tan, W., Xiu, J., Puskar, N., Fonck, C., Lester, H.A., and Feldman, J.L. (2008). $\alpha 4^*$ nicotinic receptors in preBotzinger complex mediate cholinergic/nicotinic modulation of respiratory rhythm. *J. Neurosci.* 28, 519–528.
- Tapper, A.R., McKinney, S.L., Nashmi, R., Schwarz, J., Deshpande, P., Labarca, C., Whiteaker, P., Marks, M.J., Collins, A.C., and Lester, H.A. (2004). Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science* 306, 1029–1032.
- Tapper, A.R., McKinney, S.L., Marks, M.J., and Lester, H.A. (2007). Nicotine responses in hypersensitive and knockout $\alpha 4$ mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice. *Physiol. Genomics* 31, 422–428.
- Wathey, J.C., Nass, M.M., and Lester, H.A. (1979). Numerical reconstruction of the quantal event at nicotinic synapses. *Biophys. J.* 27, 145–164.
- Whiteaker, P., McIntosh, J.M., Luo, S., Collins, A.C., and Marks, M.J. (2000). [125 I]- α -Conotoxin MII identifies a novel nicotinic acetylcholine receptor population in mouse brain. *Mol. Pharmacol.* 57, 913–925.
- Wooltorton, J.R., Pidoplichko, V.I., Broide, R.S., and Dani, J.A. (2003). Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in midbrain dopamine areas. *J. Neurosci.* 23, 3176–3185.
- Zhang, H., and Sulzer, D. (2004). Frequency-dependent modulation of dopamine release by nicotine. *Nat. Neurosci.* 7, 581–582.
- Zhou, F.M., Liang, Y., and Dani, J.A. (2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. *Nat. Neurosci.* 4, 1224–1229.
- Zhuang, X., Oosting, R.S., Jones, S.R., Gainetdinov, R.R., Miller, G.W., Caron, M.G., and Hen, R. (2001). Hyperactivity and impaired response habituation in hyperdopaminergic mice. *Proc. Natl. Acad. Sci. USA* 98, 1982–1987.
- Zoli, M., Moretti, M., Zanardi, A., McIntosh, J.M., Clementi, F., and Gotti, C. (2002). Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J. Neurosci.* 22, 8785–8789.